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ABSTRACT

In this study, we aimed to validate existing plasma assays to measure biomarkers for maternal signalling in milk and saliva of lactating sows. These biological samples are minimally invasive to the animal and could give a physiological profile of maternal qualities available to their piglets. Sows were farrowed in a zero-confinement system, and their colostrum and milk samples were manually collected during naturally occurring let-downs (i.e. not induced) over the lactation period. Saliva sampling involved sows voluntarily accepting cotton buds to chew without restraint. Commercial kits designed for blood plasma were tested, and any modifications and results are given. We successfully measured total protein, cortisol, tumour necrosis factor-α (**TNF-α**) and oxytocin in pig milk and saliva and immunoglobulin G (**IgG**) in pig milk samples. We were unsuccessful at measuring relaxin and serotonin in these biological samples. We observed higher levels of biomarkers in milk than in saliva. The measurement of TNF-α in pig milk for the first time revealed increased levels with larger litters. This development will allow more detailed understanding of biomarkers in milk. There was also evidence that the minimally invasive technique of using saliva sampling did not interrupt natural oxytocin production around parturition.

Implications

The validated protocols in this study will allow researchers a greater breadth in biological sample analysis when studying pig health and welfare. We focused on minimally invasive sampling techniques for saliva and colostrum/milk to decrease the potential negative impact of sampling on the sow. In addition, repeated sampling over time will allow a greater understanding of the natural variations seen in the analytes measured around farrowing in sows.

Introduction

Biomarkers are an important tool for measuring the physiological and disease state of an individual and can be measured in various biological fluids (Franco-Martínez et al., 2020). Although blood is often used, sampling can be associated with increases in serum steroid biomarkers (Vachon, 2001) therefore non-invasive sampling may provide a reflection of an uninterrupted, yet current physiological state of the individual animal across multiple time points. The development of reliable and robust biomarker assays plays an important role in refining methods (**3Rs**), whether for research or for medical or veterinary practice. For example, the validation of saliva and hair cortisol (Meyer and Novak, 2012) and faecal metabolites of cortisol (Palme et al., 1996) has tremendously reduced the need for blood sampling, which is invasive and often stressful.

Minimally invasive sampling is particularly relevant during the sensitive nursing period in mammals, where milk is present as an additional bioactive body fluid (Rani et al., 2017). It transfers nutrients to neonates, as well as other biofactors relating to the immune and endocrine systems. As the neonate matures, the qualities and composition of these factors change over time (Hurley, 2015).

The most notable biofactors present are immunoglobulins (**IgGs**), transferring maternal immune molecules which stimulate maturation of the offspring's immune system (Dzidic et al., 2018). Piglets are dependent on the IgGs contained in colostrum and milk as the porcine epitheliochorial placenta does not allow the passage of antibodies and immune cells from sow to foetus (Wagstrom et al., 2000; Bandrick et al., 2008).

Biofactors are released in milk in response to stimulation of the mammary gland, resulting in neuro-hormonal reflex of milk

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ejection (Castrén et al., 1993; Gilbert et al., 1994). The neuropeptide oxytocin is a modulator of social behaviours in the mother via the olfactory bulb (Wacker and Ludwig, 2012). Oxytocin has an important role in mother-offspring communication and bonding (Kendrick, 2000; Insel and Young, 2001), and is related to long-term development and cognitive function in offspring (Scatliffe et al., 2019). Other transferred biomarkers include cytokines, such as pro-inflammatory tumour necrosis factor-α (**TNF-α**), which are necessary mediators, directing neonatal inflammatory responses. Their balance is important for protection against or susceptibility to infection (Clark, 2007). Other signals about the environment, including signals for danger, may also be transferred. For example, when the dam experiences stress during certain phases of the pregnancy, it is possible for glucocorticoids, such as cortisol, to be transferred to the embryo (Kapoor et al., 2006; Harris and Seckl, 2011), especially if the maternal stress experienced surpasses certain thresholds. Cortisol is often measured in blood (Saco et al 2003), faeces (Palme et al., 1996) and saliva (Ruis et al., 1997) but has also been detected in the milk of various mammals including rhesus macaques (Hinde et al., 2015) and dairy cows (Gellrich et al., 2015; Sgorlon et al., 2015).

This study measured various biomarkers in saliva and milk of sows. Pigs are increasingly used as a research species in both veterinary and translational human research due to their physiological and genomic similarities (Rothschild and Ruvinsky, 2011). The measurement of some biomarkers has been well validated for pigs, such as blood cortisol (Saco et al., 2003), salivary cortisol (Ruis et al., 1997), faecal cortisol metabolites (Palme et al., 1996), blood oxytocin (Gilbert et al., 1994) and IgG in milk (Klobasa et al., 1987). There is insufficient information however on the measurement of salivary TNF-α, milk cortisol and milk oxytocin. Although TNF-α has been measured in pig saliva before, it was in response to experimental injections of lipopolysaccharides (Llamas Moya et al., 2006) rather than an observation of normal levels over time as proposed here. The measurement of peripheral oxytocin in biological samples such as saliva (Valstad et al., 2016) has only recently been validated in pigs (López-Arjona et al., 2020; Lürzel et al., 2020). Therefore, the measurement of biomarkers in pigs still requires further investigation (Llamas Moya et al., 2006; Lürzel et al., 2020), especially for individual differences and changes in saliva across different life stages. Commercial kits which have not been tested with milk samples require validation, which is sometimes lacking from published work, including validation across multiple time points. Other factors such as circadian rhythms (Ruis et al., 1997; Nakao, 2014) and metabolism (Parrott and Mission, 1989) may affect when sampling is carried out, as well as the sampling method itself.

The aim of this study was to validate methods for quantifying biomarkers in sow saliva and milk, as a minimally invasive technique for investigating natural ranges in mothers and potential levels transferred to piglets. We hypothesised that sows' salivary biomarker levels would correlate with those found in the sow's milk.

Material and methods

Experimental design

Nineteen Landrace × Large White sows and their piglets were studied from entry into the farrowing (parturition) accommodation to weaning at the research pig unit of Scotland's Rural College (SRUC), Midlothian, Scotland. The study consisted of two batches (i.e. farrowing groups) whereby batch 1 (**B1**) (n = 10 sows) was used to refine novel data collection techniques for physiological sampling and batch 2 (**B2**) (n = 9 sows) underwent the refined protocols. Sows were primiparous and multiparous with an average parity of 3.95 ± 1.54 (SD), range (1–6). Sows were housed in zero-confinement PigSAFE pens in which they have freedom of movement (Baxter et al., 2015). During piglet handling for data collection, the sows were temporarily locked in the feeding stall located within the pen. A total of 246 piglets were live-born (B1: 120, B2: 126), with an average litter size of 11.6 ± 1.80 (mean ± SD, range 9–14).

Sampling time points: Milk

Sow colostrum was collected 12 hours after the birth of the last piglet (D0); then, colostrum/milk samples were collected at the first available natural let-down (following the return of the piglets after their data collection) on days (**D**) 3, 5, 10, 14 and 21 post partum.

Sampling time points: Saliva

Sow saliva samples were collected 4 days before farrowing (D-4) (based on the due date calculated 115 days from serving). Mean (±SD) day of gestation prefarrow saliva was sampled was 113.64 days (±0.48). Subsequent sampling at 12 hours postpartum (D0), and thereafter 15 minutes preremoval of piglets for piglet data collection (**PRE**) and 15 minutes postreturn of piglets (**POST**) on sD 3, 5, 10, 14 and 21 postpartum.

Sow saliva sampling

All sows were offered via gentle approach minimising disruption (either outside or inside the pen), by a familiar handler two large cotton buds (Millpledge Veterinary, Clarborough, Nottinghamshire, UK) on which to chew for approximately 30 seconds or until saturated with saliva. The cotton buds were placed into prelabelled Salivette tubes (SARSTEDT AG & Co., Nümbrecht, Germany), which were sealed and centrifuged for five minutes at 1400*g*. The supernatant was pipetted into prelabelled 1.5 ml tubes and stored at −70 °C for biomarker assays at a later date.

For B1, sows were sampled for different biomarker components across six sampling days spread throughout lactation (D-4, D0, PRE and POST D5, D10, D14 and D21 postpartum). For B2, saliva sampling occurred across seven sampling days throughout lactation, D-4, D0, then PRE and POST D3, 5, 10, 14 and 21 postpartum (with POST results not being significantly different from BASAL and therefore being henceforth disregarded from study leaving seven sampling points).

Colostrum and milk sampling

An experienced handler manually obtained colostrum and milk samples from the sow (without oxytocin injection induction), while lying laterally, during naturally occurring suckling bouts when the piglets were returned to the sow after piglet data collection. As a natural response, most sows allowed the piglets to suckle quickly after reunion. The experienced handler waited for an established suckling bout (and hence milk let-down) before quietly entering the pen keeping a low profile, then sampled from as many teats as possible during the point of milk let-down (approximately 20 seconds), utilising unoccupied teats and briefly removing piglets from an occupied teat in order to do so. Signs of milk ejection included a change in piglet behaviour from massaging of teats and non-nutritive suckling to a focused rapid suckling motion with the mouth, clear signs of milk consumption (i.e. nutritive suckling) and milk residue around the mouth. The handler did not usurp piglets considered to be vulnerable (i.e. low BW) or piglets that had just been blood sampled. Large rimmed plastic beakers (50 ml Thermo-

Fisher Scientific) were used to ensure easy collection of around 3 ml of milk in total. Samples were immediately pipetted into smaller Eppendorf tubes, as approximately 100 μl of skimmed milk is required per assay and stored at −70 °C until later analysis.

Assay validation methods

Refinements and validations of assays were conducted with B1 samples. This determined not only the sampling protocols but also which biomarkers (analytes) were then measured in B2. This included suitability of analyte for sample type (saliva, milk), dilutions specific for each analyte and sample time point. All assays (unless otherwise specified) were read at 450 nm using a spectrophotometer with Thermo SkanIt software (Multiskan, Thermo Scientific, USA). All assays included positive quality controls (QCs) and assays were only accepted if R^2 was above 0.98, curve fit percentage recovery was within the 70–130% range and intra-plate and inter-plate CV% had a threshold for acceptance of below 20%. In addition, individual sample results were only accepted if they had a CV% of less than 20%. We detail below all biomarkers tested and those which were unable to be confidently validated in sow milk and/or saliva and the final conditions used for analysis.

Sow saliva biomarkers: Steroids (cortisol)

For batch one, cortisol was measured with two different assays. One subset of undiluted saliva samples from five sows at four sampling points D0, D5, 10, and 21 (n = 20) measured cortisol (ng/ml) using the steroid/thyroid panel (Millipore #S90003) which also detects estradiol, progesterone, T3, and T4 (data not shown). It was read on the BioPlex 200 (BioRad). QC recoveries were all within range. In addition, we measured salivary cortisol (ng/ml) by a second method in a further 35 undiluted saliva samples from the same five sows in seven additional sampling points (D0-F, D0, POST D5, PRE and POST D10 and D14). Salivary cortisol (ng/ml) in these 35 samples were measured with an ELISA (ALPCO #11-CORHU-E01-SLV). In batch two, salivary cortisol (ng/ml) was measured using the single ELISA method (ALPCO) of B1. QC was 112.00% and 87.77% for B1 and B2, respectively. Intra-plate was <8% and inter-plate was <15% for both batches.

Tumour necrosis factor-α

TNF-α (pg/ml) was measured in 99 saliva samples (B1) and 117 samples (B2) with an ELISA (R&D systems, Duoset #DY690B). No dilution was used, and the manufacturer's instructions were followed. QC recovery was 121.8% and 118.4% for B1 and B2, respectively. Intra and inter-plate CV% were <8% for all batches.

Oxytocin

Salivary oxytocin (pg/ml) was measured in 99 (B1 and B2) undiluted samples using an ELISA (Cusabio, #CSB-E12062p). QC recoveries were 105.86% and 122% for B1 and B2, respectively. Intra-plate CV% was <12%, and inter-plate CV5 was <15%. For final conditions of assays for biomarkers in sow saliva, see Table 1.

Sow milk biomarkers

Milk samples were allowed to thaw on ice then centrifuged at 13 000 rpm for 3 mins. Fat layers were measured as a proportion of total content, removed (skimmed) and milk further aliquoted for further milk analysis. Due to the natural changing composition of colostrum/milk, appropriate dilutions of milk for each analyte and time point needed to be determined in B1.

Total protein content

The total protein of colostrum/milk was first analysed to estimate changing composition over sampling points. This then informed the dilutions required for analysing IgG concentrations. For total protein content (mg/ml), skimmed milk from B1 was diluted at 1:100 and 1:1 000 in phosphate buffered saline. As 1:100 was the most appropriate for the range of samples, this dilution was used for all B2 samples. Milk total protein content was measured using Quick Start™ Bradford Protein Assay Bradford reagent (BioRad #5000202) following the microplate instructions. These results helped guide the dilution used for milk IgG measurements. The dilution for colostrum or milk sampled at D0 was 5 000, at D3 1 000, at D5 100, at D10 50 and at D14 and D21 the dilution was set to 20.

Immunoglobulin G

Following dilution of B2 milk only, IgG (mg/ml) was measured using Pig IgG ELISA (Cusabio cat #CSB-E06804p) following manufacturer's instructions. QC was 103.0% and 113.55% for B1 and B2, respectively. Intra-plate CV% was <8% for both B1 and B2; inter-plate-CV5 was <10% for both batches.

Steroids (for cortisol)

Cortisol measurements in skimmed milk were carried out as for saliva (see sow saliva biomarker section).

Tumour necrosis factor-α

The TNF-α concentration in skimmed milk was measured in 48 samples in B1 and 54 samples in B2 using an ELISA (R&D systems, Duoset #DY690B). No dilution was used, and the manufacturer's instructions were followed. QC recovery was 103.65% and 102.37% for B1 and B2, respectively. For both batches, intra-plate CV% was <13% and inter-plate CV% was <8%.

Oxytocin

Undiluted skimmed milk was used to measure oxytocin from 54 samples (from each batch) using an ELISA (Cusabio, #CSB-E12062p). QC recovery was 113.53 and 106.09% for B1 and B2, respectively. Intra-plate CV% was <5% and <10% for both batches. For final conditions of assays for biomarkers in sow milk, see Table 1.

Biomarker assays that could not be validated

Commercial kits were used to detect biomarkers. Each kit has a list of biological samples that have been validated by the company (Table 1). We investigated whether kits could also be used to detect biomarkers previously unvalidated in sow milk and saliva. Although we had some success (see sections above), there were a couple of kits that were too specific to be used for additional sample types. One example is the serotonin assay, which is suitable for use with platelets, serum, citrate plasma and urine. We attempted to measure serotonin (ng/ml) in undiluted skimmed milk and saliva samples using an ELISA (Enzo, ADI-900-175). There were a number of problems with samples and QCs on this plate, and therefore, no confident results were produced for saliva or milk. Relaxin (pg/ml) was measured in undiluted skimmed milk samples using Pig RLN/Relaxin ELISA (LSBio cat # LS-F12488) (validated for use in plasma/serum), but although the kit worked (QC within acceptable range), the samples were all below the assay's level of

Table 1
Commercial kits used to successfully measure biomarkers in sow samples (milk, saliva) with specific conditions used in this study. Note: steroid multiplex panel conditions not included as not used for batch 2.

Analyte	Commercial kit information			Conditions used in this study	
	Kit info.	Samples validated by company	Range/unit	Sample tested	Sample dilution
Total Protein	Quick Start™ Bradford Protein Assay, BioRad (5000202)	Sample solution	0.125–2 mg/ml	Milk	100
TNF-α	Porcine TNF-alpha DuoSet ELISA R&D systems (DY690B)	Serum, plasma, cell culture supernates,	31.3–2 000 pg/ml	Saliva	Undiluted
				Milk	Skimmed, undiluted
IgG	Pig Immunoglobulin G ELISA CUSABIO (CSB-E06804p)	Serum, plasma, cell culture supernates, tissue homogenates	0.586–150 μg/ml	Milk	Skimmed, dilutions per sampling day
				D0	5 000
				D3	1 000
				D5	100
				D10	50
				D14	20
				D21	20
Oxytocin	Pig oxytocin, OT ELISA kit CUSABIO (CSB-E12062P)	Serum, plasma, tissue homogenates	2.5–100 pg/ml	Saliva	Undiluted
				Milk	Skimmed, undiluted
Cortisol	ALPCO (11-CORHU-E01-SLV)	Saliva	0.27–200 ng/ml	Saliva	Undiluted

TNF-α: tumour necrosis factor-α, IgG: immunoglobulin, D: day.

detection. Additional methods to detect and quantify these biomarkers in saliva and milk are therefore needed.

Statistical analyses

Data were analysed using SAS software, version 9.4 (Copyright© 2016 SAS Institute Inc., Cary, NC, USA). The statistical models were assessed for the assumptions of homogeneity of variance (using Levene's Test) and normal distribution of the model residuals (by inspecting residual plots). Models were adjusted for multiple comparisons by the Tukey-Kramer adjustment. Values are presented as LSmeans with SE, unless stated otherwise (as means with SD).

Analysis of sow saliva and milk analytes

Milk. The difference between sampling days for milk fat and milk protein was analysed in linear mixed models (MIXED Procedure) with fat/protein as response variable, the sampling day as predictor variable and the sow specified as repeated observation (included as subject to account for repeated observations).

Saliva. The differences between the PRE and POST saliva values were assessed using paired t-tests. Based on no significant difference (described in the Results), only the PRE (before piglets were removed) saliva samples were used in the further analysis, as the PRE samples more closely resemble a standard sampling procedure.

The saliva sample of D-4 was compared to the saliva collected on postfarrowing days as potential methodological refinement, and as there is no milk prefarrowing to compare the sample to. In linear mixed models, with the analyte as response variable, sampling day was included as predictor variable and batch and sow as random effects. In the posthoc comparisons, D-4 was compared to the other sampling days.

Comparison of milk versus saliva

For the comparison of analytes across sample types (saliva versus milk), only the PRE samples for D0, 5, 10, 14 and 21 were retained. In model 1, the association between saliva and milk samples was analysed in a linear mixed model (MIXED procedure) for each analyte, with the saliva sample as response variable and the milk sample and sampling day as predictor variables. Batch and sow were included as random effects with sow specified as subject to account for repeated observations (i.e., sow as experimental unit).

In model 2, the saliva and milk samples were compared in more detail by analysing the data in a similar mixed model, but with the analyte (cortisol, TNF-α, oxytocin) as response variable, and the sample type (milk versus saliva), sampling day, and their interaction (sample type × sample day) as predictor variables. Batch and sow were included as random effects, with sow being specified as the subject. Differences were inspected through posthoc comparisons, using the Tukey-Kramer adjustment for multiple comparisons.

Effect of sow characteristics on analytes

The effect of sow parity, total number of live-born piglets and batch on the analytes was assessed by analysing the average value per analyte per sow as a response variable in a General Linear Model (GLM), with the aforementioned variables included as predictor variables, including the interaction between litter size (number of live-born piglets) and batch.

Results

Success and reliability of assays

Average sample CV% for all analytes is given in Table 2. There were some differences in range and sensitivity of the kits, such as a lower average sample CV% in single cortisol ELISA (12%) as compared to the Thyroid multiplex (19%) (Supplementary Table S1).

Sow saliva

The average values of the analytes in PRE and POST saliva did not significantly differ from each other (for all analytes, $P > 0.10$). Therefore, only the PRE value is presented here.

Saliva at D-4 was compared to the postfarrowing days. Salivary oxytocin (pg/ml) was significantly higher on D-4 (43.51 ± 5.10) as compared to D10 (20.19 ± 4.93; $P = 0.001$), D14 (18.69 ± 4.93; $P < 0.001$) and D21 (19.35 ± 4.51; $P < 0.001$), but did not differ from D0 (50.13 ± 7.76; $P = 0.47$) or D5 (41.83 ± 4.78; $P = 0.81$). The values of salivary cortisol and TNF-α, on D-4, did not significantly differ from the values on any of the other days ($P > 0.10$). For average concentrations of analytes measured in saliva, see Table 3.

Table 2
Average sample CV% for all analytes for sow saliva and milk samples. *denotes multiplex thyroid panel, batch one (B1) only. ND denotes no data.

Analyte (unit)	Milk: Average sample CV%		Saliva: Average sample CV%	
	Batch 1	Batch 2	Batch 1	Batch 2
Total protein (mg/ml)	17.6	7.8	ND	ND
TNF-α (pg/ml)	12.7	11.1	1.4	1.2
IgG (mg/ml)	15.1	14.8	ND	ND
Cortisol (ELISA) (ng/ml)	6.5	ND	14.9	7.9
Oxytocin (pg/ml)	16.9	14.7	16.1	7.9
Thyroid panel-cortisol* (ng/ml)	20.4	ND	19.2	ND

TNF-α: tumour necrosis factor-α, IgG: immunoglobulin.

Sow milk

Sows' milk contained on average 12.27 ± 5.17 (SD) % fat (range 3.2–32%) and 54.89 mg/ml ± 29.38 protein (17.3 –159.7 mg/ml). The amount of fat in the milk was significantly higher on day 3 postpartum but was stable across the other days (Fig. 1; $F_{5,67} = 5.69$; $P < 0.001$). The amount of protein was highest on the day of farrowing (Fig. 1; $F_{5,65} = 8.27$; $P < 0.001$) whereas it did not significantly differ across the remaining days of lactation (Fig. 1). An expected trend of IgG was seen in the milk for B2 sows, with individual variation, with highest concentrations observed at farrowing (average 341.2 ± 405.4 (SD) and a decline in the days thereafter (Fig. 2). For average concentrations of cortisol, oxytocin and TNF-α measured in milk, see Table 3.

Comparison of sow saliva and milk analytes

The values for saliva were not significantly associated with the values in the milk, for cortisol ($P = 0.48$), oxytocin ($P = 0.32$) and TNF-α ($P = 0.58$) (model 1). While not associated with each other (model 1), the values of the analytes in the milk were overall higher than the saliva samples, which was significant for oxytocin and TNF-α and a tendency for cortisol (Fig. 3B and C) (analysed using model 2).

The values for cortisol (milk and saliva together; model 2) differed between sampling days ($F_{4,61} = 4.77$; $P = 0.002$) with the value at D0 (33.39 ± 4.80 ng/ml) being significantly higher than on D10 (20.13 ± 5.63 ng/ml) and D21 postpartum (21.57 ± 4.75 ng/ml) (Fig. 3A). Cortisol values did not show an interaction between the sample type and sampling day ($P > 0.10$). The oxytocin level on D0 was significantly higher than on any of the other sampling days (Fig. 3B; $F_{4,123} = 7.10$; $P < 0.001$). There was a significant interaction between sample type and sampling day ($F_{4,123} = 4.37$; $P = 0.003$), with posthoc differences between the saliva and milk sample on D14 (saliva: 18.16 ± 4.86; milk: 42.59 ± 4.71; $P = 0.01$) and D21 (saliva: 19.35 ± 4.44; milk: 40.84 ± 4.44; $P = 0.02$). The value for TNF-α did not significantly differ between sampling days ($P = 0.84$) and did not show an interaction between sampling day and sample ($P = 0.72$) (Fig. 3C). However, posthoc tests do show a significant difference for saliva

Table 3
Means with pooled SE, averaged across all sampling days, for analytes measured in sow saliva and milk (*n* indicates the total number of samples prehandling).

Biomarker	*n*	Saliva	*n*	Milk	SEp	*P*-value
Cortisol (ng/ml)	65	21.35	18	28.82	4.996	0.10
Oxytocin (pg/ml)	70	29.60	80	38.32	3.422	0.006
TNF-α (pg/ml)	31	27.91	59	343.00	73.159	<0.001

TNF-α: tumour necrosis factor-α.

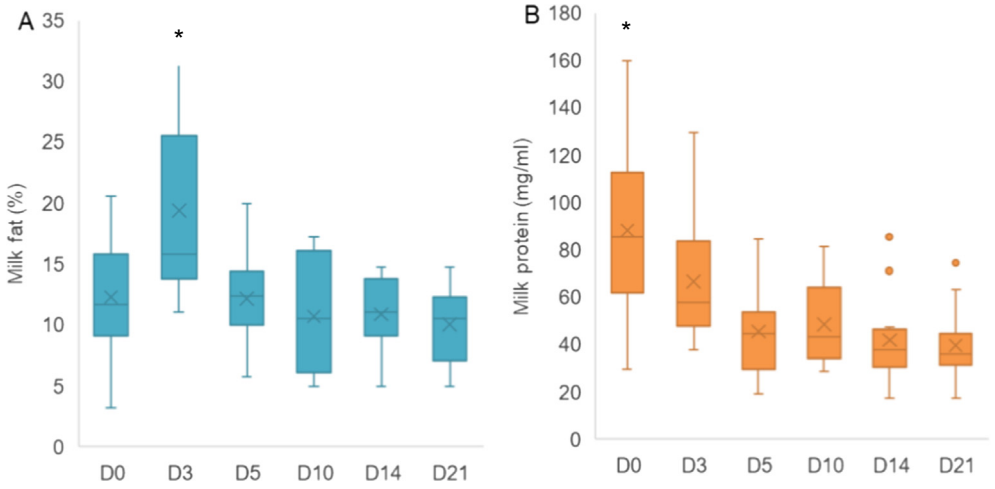

Fig. 1. (A) Sow milk fat (%) and (B) sow milk protein levels (mg/ml) across lactation, for days 0, 3, 5, 10, 14 and 21 postpartum. The number of sows per sampling day varies between 8 and 14 (average 12). The boxplot depicts the means, median (cross), minimum, maximum and SD. The asterix indicates the day that differs from the other days by $P < 0.05$.

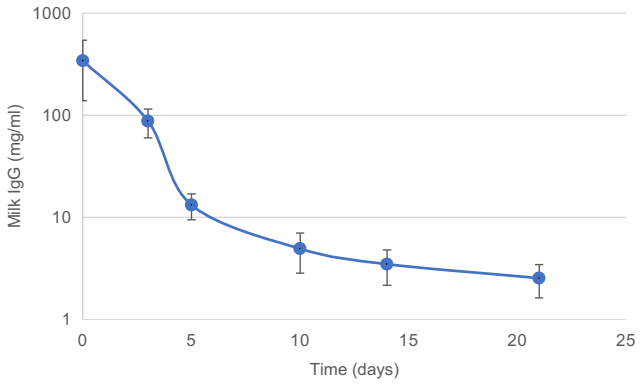

Fig. 2. Mean immunoglobulin (IgG) (mg/ml) (±SD) in sow milk between 0 and 21 days. Range min 0.18 to max 919.42 mg/ml.

versus milk samples at D5 only (saliva: 7.55 ± 103.32; milk: 373.30 ± 88.07; $P = 0.04$).

Effects of sow characteristics

Overall, the patterns of the saliva and milk analytes show a strong variation between individual sows (Fig. 3). Sow parity significantly influenced several analytes and led to higher values in older sows for saliva cortisol ($b = 4.89 \pm 9.76$; $P = 0.04$), saliva oxytocin ($b = 4.03 \pm 1.36$; $P = 0.01$), and tended to result in a higher saliva TNF-α ($b = 7.04 \pm 4.00$; $P = 0.10$). Litter size only had an effect on milk TNF-α, where more live-born piglets per litter resulted in a higher TNF-α ($b = 7.9 \pm 16.99$; $P = 0.003$), but this was influenced by an interaction between litter size and batch ($P = 0.01$) due to more extreme litter sizes in B1. Batch differences were significant for saliva TNF-α, where B1 had higher values (90.0 pg/ml) than B2 (60.4 pg/ml) ($P = 0.01$), and for milk oxytocin, where values tended to be lower in B1 (34.6 pg/ml) than in B2 (43.7 pg/ml) ($P = 0.09$), but not for the other analytes.

Discussion

This study aimed to demonstrate the potential for using novel, minimally invasive techniques to characterise important biomarkers in sow milk and saliva and thus how these biomarkers may be used to inform impact on offspring outcomes.

Validation of commercially available kits for novel biological fluids

Commercially available assay kits were successfully used to measure cortisol, IgG, TNF-α and oxytocin using a refined, novel approach aimed at reducing the disturbance to the experimental animals. We could not validate relaxin or serotonin for pig milk and saliva samples; however, we are confident the kits are suitable for other described validated samples (such as plasma). IgG has been measured in milk numerous times before (Bourne and Curtis, 1973; Klobasa et al., 1987; Wagstrom et al., 2000), and similar trends of values were observed in this study.

Use of saliva and milk as minimally invasive sample types

Importantly, the relationship identified between salivary oxytocin 4 days prefarrowing, on farrowing day (D0-F) and D5 postpartum suggests it may be possible to avoid sampling on farrowing day itself, mitigating any negative effect from interference during sampling on the mother-young bond. Milk collection from the sow is challenging and potentially dangerous for the sampler due to risks associated with maternally defensive behaviour (Marchant-Forde, 2002). In research trials attempting milk sampling, an oxytocin injection is often given to artificially stimulate milk production and therefore ease collection (rats (Freund-Mercier and Richard, 1981), sheep (Zamiri et al., 2001), pigs (Craig et al., 2019). In addition, typical accommodation for sows is the restrictive farrowing crate which facilitates easy management and safe data collection. Both these factors, however, although convenient, are known to influence natural milk production and therefore return results that do not truly reflect the natural physiological status of the mother. It is known that sows in free farrowing accommodation with an ability to more fully perform species-specific nest-building behaviour have higher oxytocin profiles than sows in farrowing crates (Yun et al., 2013). Misuse of oxytocin around farrowing and in the postnatal period can have negative effects on sow and piglet welfare (Mota-Rojas et al., 2002, 2006). This includes both the biological functions of oxytocin and obviously using it to artificially stimulate milk production is counterintuitive in a study such as ours that seeks to document natural variation in maternal biofactors. Thus, as we wanted to measure naturally occurring oxytocin in the milk, we did not use such injections but collected the milk directly from the sow at natural milk ejection during nursing bouts. To the best of our knowledge, this is the first time an intensive milk collection protocol (see colostrum and milk sampling section) has been reported in the case of freely moving sows. Designing a protocol that would allow maximal data collection with minimal disturbance was prioritised utilising highly skilled handlers. Refinement of sample collection can be supported as animal interference and sample number can be reduced without compromising information. For example, we collected PRE and POST piglet processing sow saliva samples but as no significant differences in data between these sampling points was observed, the analysis was conducted on presamples only. Future studies could reduce sample collection and adhere to a best practice approach in accordance with the 3Rs (Tannenbaum and Bennett, 2015) and ARRIVE guidelines (du Sert et al., 2020). In addition, for a number of circulating biomarkers, the levels measured 4 days before farrowing were not significantly different from individual sow levels for the first 5 days postfarrowing. This was a surprising finding that offers great potential to further refine future data collection protocols to reduce interference, particularly during the perinatal period where mother-young interactions are sensitive to disturbance. This time period would warrant further attention in future studies.

Saliva sampling in pigs is a simple procedure with pigs readily accepting and chewing on large cotton buds with little encouragement or disturbance. Thus if saliva could be used as a non-invasive measure of circulating maternal blood biomarkers and even those transferred to piglets via milk, it would be advantageous. Results from this work offer initial evidence that saliva can be a proxy method for milk biomarker composition, with no significant differences in TNF-α between saliva and milk identified. Further work is needed to look at measurements in finer detail and correlations with blood plasma concentrations.

Tumour necrosis factor-α

One interesting result observed was the increase in milk TNF-α levels with litter size. Large litter size is associated with a number of negative welfare outcomes for sows and piglets (Rutherford et al., 2013). Although TNF-α has been detected in human milk (Rudloff et al., 1992), this is to our knowledge the first time that TNF-α has been measured in pig milk. TNF-α is an inflammatory biomarker (Souza et al., 2008). It is known that TNF-α can travel from blood to saliva (Llamas Moya et al., 2006) but can also be produced by salivary gland epithelial cells (Sugawara et al., 2002). TNF-α has possible roles in ripening of the cervix in preparation

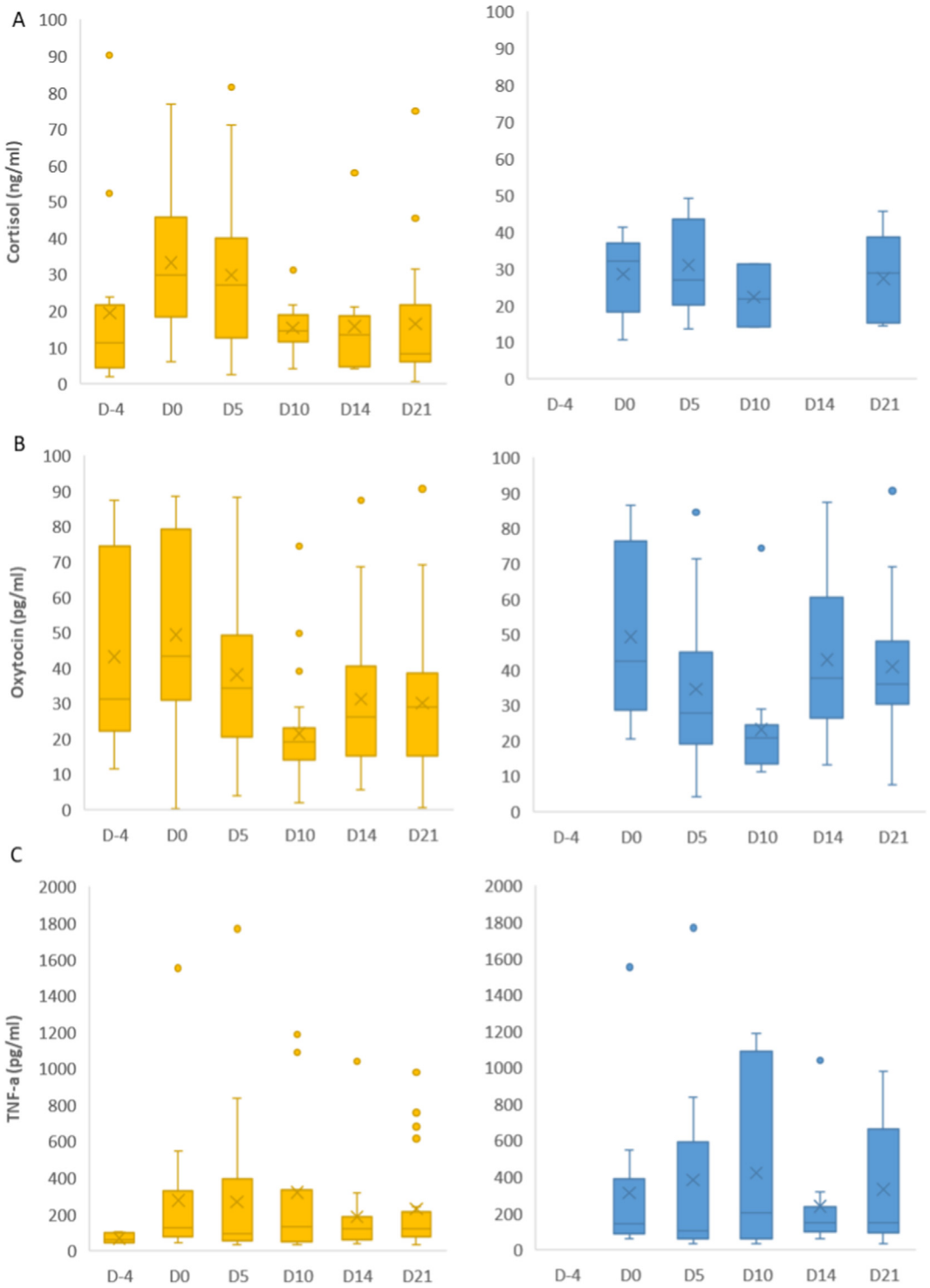

Fig. 3. Analytes from sow saliva (yellow) and sow milk (blue) for (A) cortisol, (B) oxytocin and (C) tumour necrosis factor (TNF-α) samples for days (D) 0, 5, 10, 14 and 21 postpartum. Sow saliva analytes were also analysed at D-4. The boxplot depicts the means, median (cross), minimum, maximum and SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for parturition (Chwalisz et al., 1994; Winkler and Rath, 1999), and TNF-α (along with IL-1β, IL-8) is implicated in human preterm labour and uterine infection (Romero et al., 2006). However, in our study, parturition was full term for all animals with average gestation length of 116.40 days (±1.08 SD; range 114–118). While higher milk TNF-α levels with larger litters and higher cortisol and oxytocin concentrations were observed in older, higher parity sows in this study, we cannot discount the potential cumulative effects of sow age and maternal experience. These sows, who have previously experienced pregnancy, parturition and nursing, will have more developed mammary systems, thus better maternal potential however they are more susceptible to damage. For this reason, it is not possible to understand these biomarker levels as a standalone event, they will be influenced by past experiences which will in turn affect the next experience of pregnancy and parturition. To our knowledge, inflammatory biomarkers have not been investi-

gated with respect to large litter size. This study highlights the potential for inflammatory biomarkers to be a focus of further studies investigating the impact of large litter size on welfare.

Oxytocin

There were higher oxytocin levels in milk compared to saliva; however, the directionality was similar and further work could allow a correction factor to be calculated. It was not unsurprising that milk oxytocin levels were higher given the known animal-bonding roles of oxytocin. In non-human animals mothering is hormone dependent (Feldman, 2012) with oxytocin being a key hormone, well-known to affect maternal behaviour, influencing both parturition and lactation. It has also been implicated in positive social behaviour, pleasure, and stress tolerance (for review, see Chen and Sato, 2017). Oxytocin is released during parturition and milk ejection but there is also evidence that various physical interactions can increase its release including low intensity stimulation of the skin (e.g. response to touch, stroking and warm temperature (Uvnäs-Moberg, 1998). Parental care in pigs is not characterised by overt and proactive behaviours seen in other mammalian mother-offspring interactions (e.g. licking, gathering, nuzzling, etc.) and thus opportunities for physical contact are limited, almost entirely, to occurrences of milk let-down. Milk let-down triggers oxytocin release and piglets will spend time both pre- and post-let-down massaging the sow's udder (Castrén et al., 1993). Plasma oxytocin levels are observed to increase 30 seconds before milk ejection (Ellendorff et al., 1982) and reach peak levels within 10 seconds of suckling (Algers et al., 1990). Levels of oxytocin present during this time vary between sows (Yun et al., 2014) and could serve as a proxy measure of mothering ability. This study did however not compare minimally invasive samples with blood levels of biomarkers such as previous studies in cows (Nakajima et al., 1997) but we now have the tools to do so.

Conclusion

This study focused on measuring natural individual variation in minimally invasive perinatal biomarkers. These initial results direct us to investigate further the impact of perinatal biomarkers on sow productivity and longevity. Levels of biomarkers were significantly higher for oxytocin and TNF-α in milk than saliva in this study, although not associated. This investigatory study into laboratory assay validation has resulted in measurement of biomarkers in several minimally invasive sample types in the sow which can be used to explore how they reflect other biological samples, such as blood.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100369.

Ethics approval

The experiment was reviewed and approved by SRUC's Animal Welfare Ethical Review Board (ED AE 23-2016) and conducted under Home Office Licence authority (PPL 60-4330).

Data and model availability statement

Please contact corresponding author for data and model requests.

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Declaration of interest

Authors no declaration of interest.

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